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Inhibition by Zinc of Hemolysis Induced by Bacterial and Other Cytolytic Agents

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Zinc, cupric, and cadmium ions, in that order of effectiveness, inhibited lysis of washed, rabbit erythrocytes by the toxic bacterial product aerolysin. Hemolysis induced by a variety of other lytic agents was also inhibited by Zn^{2+} in approximately the same concentration as that, 0.33 mM, needed to inhibit aerolysin-induced hemolysis. Zinc ions did not inhibit osmotic lysis. Inhibition requires the continued presence of Zn^{2+} and apparently involves a readily reversible binding of Zn^{2+} to the cell surface, which, it is postulated, is accompanied by a reversible alteration in the state of the lipid bilayer.

In studying the properties of aerolysin, a hemolytic and lethal protein derived from *Aeromonas hydrophila*, we observed that lysis of washed erythrocytes by this agent was completely inhibited by inorganic salts of zinc. Subsequently, the effect of a number of other divalent cations was studied, as was the effect of zinc ions in a variety of hemolytic systems. The results of these and other experiments bearing on the mechanism of inhibition are presented.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are: Tris, 0.01 M tris(hydroxymethyl)aminomethane, pH 7.2; TS, Tris containing 0.145 M NaCl; TSG, TS containing 0.2% gelatin; and HU, hemolytic units, as defined earlier (3).

Hemolytic agents and hemagglutinins. Aerolysin (3), streptolysin S (1), staphylococcal alpha-toxin (5), staphylococcal beta-toxin (4), and *Clostridium perfringens* alpha-toxin (4) were prepared as described in the references indicated. Streptolysin O and *C. perfringens* theta-toxin were gifts from S. P. Halbert and C. Smyth, respectively. Ricin A and wheat germ agglutinin were kindly supplied by J. Oppenheim. Saponin was from Merck and Co. (Rahway, N.J.), lysolecithin from Sigma Chemical Co. (Saint Louis, Mo.), and Triton X-100 from Research Products International Corp. (Elk Grove Village, Ill.)

Hemolytic activity. Hemolytic activity was estimated as described elsewhere (3). For *C. perfringens* theta-toxin and streptolysin O, titrations were carried out after activation with cysteine. For *C. perfringens* alpha-toxin and staphylococcal beta-toxin, TSG was modified to contain 5 mM calcium chloride and 10 mM $MgCl_2$, respectively. For these two lysins, sheep erythrocytes were substituted for rabbit erythrocytes, and "hot-cold" conditions of incubation (4) were used. "Percent hemolysis," when used as a measure of lytic activity, means 100 times the ratio of hemoglobin liberated from erythrocytes to total hemoglobin of erythrocytes, as measured at 540 nm on supernatants.

RESULTS

Inhibition of aerolysin-induced hemolysis by zinc ions. Complete inhibition of hemolysis by 1 mM zinc acetate is shown by curve D of Fig. 1, as contrasted with nearly complete lysis in the absence of zinc acetate seen in curve A. When zinc acetate was present only during the first 30 min and then removed by centrifugation, but with aerolysin added back, lysis proceeded as if the salt had not been present (curve B). When both aerolysin and zinc acetate were removed after 30 min, an insignificant amount of lysis occurred. The reaction mixture used for curve B was centrifuged at 30 min, and the supernatant fluid was dialyzed overnight to remove zinc ions and then added to a fresh quantity of erythrocyte suspension. Nearly full hemolytic activity was recovered. The results indicate that inhibition (i) requires the continued presence of zinc acetate, (ii) is not due to irreversible inactivation of aerolysin by zinc acetate, and (iii) is not due to irreversible alteration of erythrocytes by zinc acetate. In this type of experiment, and in others, the effects of equimolar concentrations of zinc acetate and zinc chloride were identical.

Capacity of various divalent cations to inhibit aerolysin-induced hemolysis. Divalent cations decreasing in concentration in steps of about 30%, between 5 and 0.05 mM, were examined for capacity to inhibit aerolysin-induced hemolysis. The test amount of aerolysin was 10 HU, and the end point was 50% lysis. The results (Table 1) show that salts only of Zn, Cu, and Cd inhibited in the range tested. It is possible that Cu^{2+} is potentially more effective than indicated, because Cu^{2+} is known to interact relatively strongly with the buffer used (7).

Effect of zinc ions in various hemolytic systems. Using conditions similar to those of the

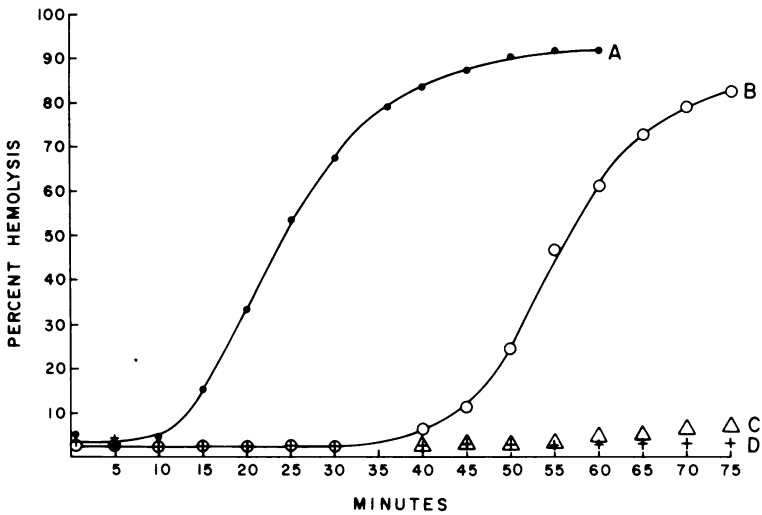

FIG. 1. *Inhibition of aerolysin-induced hemolysis by zinc acetate. A, Course of hemolysis in presence of 30 HU of aerolysin. Initial reaction volume was 40 ml. B, As A but in the presence of 1 mM zinc acetate for first 30 min. At 30 min, the mixture was centrifuged, and the cells were suspended in the same milieu as in A. C, As B for first 30 min. After 30 min, neither zinc acetate nor aerolysin was present. D, As A but with zinc acetate and aerolysin present during entire experiment.*

TABLE 1. *Capacity of various divalent cations to inhibit aerolysin-induced hemolysis*

Cation[a]	Concn (mM) inhibiting 90% of test amount of aerolysin (10 HU)[b]
Zn^{2+}	0.33
Cu^{2+}	0.60
Cd^{2+}	5
Ni^{2+}	>5
Co^{2+}	>5
Fe^{2+}	>5
Mn^{2+}	>5
Mg^{2+}	>5
Ca^{2+}	>5

[a] All were tested as chlorides except Cu^{2+}, which was the sulfate.

[b] 10 HU causes complete hemolysis.

TABLE 2. *Inhibition by zinc ions of lysis induced by diverse hemolytic agents*

Lytic agent	Concentration (mM) of Zn^{2+} inhibiting 90% of test amount of lytic agent (10 HU)[a]
Aerolysin	0.33
Streptolysin S	0.22
Streptolysin O	0.28
Lysolecithin	0.33
Saponin	0.31
Staphylococcal alpha-toxin	0.30
Staphylococcal beta-toxin	0.15
C. perfringens alpha-toxin	0.15
C. perfringens theta-toxin	0.28
Triton X-100	0.35

[a] 10 HU causes complete hemolysis.

preceding experiment, lysis-inhibiting capacity of zinc ions was estimated in a variety of hemolytic systems. The results (Table 2) show that, in all instances, lysin was inhibited by zinc chloride in concentrations between 0.15 and 0.35 mM. In the absence of further data, the differences in concentrations needed for inhibition are not considered significant. In contrast to these results, lysis of erythrocytes by hypotonic solutions of NaCl was found not to be appreciably inhibited by zinc in concentrations up to 5 mM.

Solutions of each lytic agent (except saponin, which is dialyzable), containing about 20 HU/ml and 1 mM zinc ions, were placed at 37 C for 10 min and then dialyzed overnight against TS to remove zinc ions. In each case, 50 to 100% of the initial lytic activity was restored by dialysis. Hence, the lytic agents were not irreversibly inactivated by zinc ions.

Reversible binding of zinc ions to erythrocytes. To approximately 4.1×10^{9} washed rabbit erythrocytes was added zinc acetate to a concentration of 0.3 mM. The reaction volume was 3 ml, and the milieu was TS. After 5 min at 30 C, the mixture was centrifuged at $3,000 \times g$ for 10 min. The supernatant fluid was separated from the pellet as completely as possible, and the pellet was suspended in 0.3 ml of TS, after which 2.7 ml of distilled water was added. The solution derived from the pellet, as well as the original supernatant were analyzed for zinc

according to Zak et al. (13). Comparable mixtures in which either erythrocytes or zinc ions were omitted were also set up and analyzed. The erythrocytes in an additional reaction mixture, identical to that described, were eluted with 3 ml of TS, and the pellet and eluate were analyzed. The results (Table 3) are interpreted as meaning that erythrocytes are capable of binding an appreciable quantity of zinc. Parenthetically, the amount of zinc found in the pellet of erythrocytes not exposed to Zn^{2+} (6 μg) is closely similar to the amount of zinc (5.6 μg) reported to be present in a comparable volume of packed human erythrocytes (10).

Effect of hemagglutination on hemolysis. It was observed that inhibitory concentrations of zinc ions caused some of the erythrocytes to form small aggregates. Upon removal of zinc ions by centrifugation and resuspension of the pellet in zinc-free medium, the clumps dispersed, indicating that hemagglutination by zinc ions, like hemolysis inhibition, is reversible. To evaluate the effect of hemagglutination itself on hemolysis, we examined the effect of ricin and wheat germ agglutinin on lysis by streptolysin S and of wheat germ agglutinin on lysis by staphylococcal alpha-toxin. The results (Table 4) show that somewhat less lysis occurs in the presence of hemagglutinating agent than in its absence.

TABLE 3. *Results of zinc analyses*[a]

	Amt of zinc (μg)
Added to 4.1 × 10^{9} erythrocytes	59
Found in supernatant of reaction mixture	51
Eluted from pelleted erythrocytes	10
Found in pellet after elution	7.2
Found in pellet of erythrocytes not exposed to Zn^{2+}	6.0

[a] See text.

TABLE 4. *Effect of hemagglutination on hemolysis*

Lytic agent (4 HU)	Addition (μg/ml)	% Hemolysis
Streptolysin S	None	93
	Ricin A (0.5)	68
	(2.5)	47
	Wheat germ agglutinin (5)	78
Staphylococcal alpha-toxin	None	87
	Wheat germ agglutinin (5)	78

DISCUSSION

The data leave no doubt as to the capacity of zinc ions to inhibit erythrocyte lysis. The mechanism of the inhibition, however, is less obvious. The results show that inhibition occurs only while zinc ions are present in the system, and that zinc ions, in the concentrations used, combine irreversibly neither with erythrocytes nor, apparently, with the lytic agents. There remain the possibilities that zinc ions combine reversibly with either erythrocytes, the lytic agents, or both. The analytical measurements demonstrate that zinc ions are taken up by erythrocytes. Attachment to the cell, presumably to the cell surface, however, is a loose one, because the zinc readily dissociates from the erythrocytes when they are suspended in zinc-free solution. Shedding of zinc is accompanied by a return of the erythrocytes to the unsuppressed state. Although the possibility of reversible inactivation of lytic agent has not been excluded, the simplest hypothesis consistent with the experimental findings is that inhibition is a consequence of reversible binding of zinc to the cells.

The principal mechanism of lysis inhibition presumably involves a labile blocking, by zinc ions, of lysin binding sites on the cell surface. The blocking may be either direct or indirect, the latter as a consequence of hypothetical conformational changes induced by the metal ions. It is known that some of the lysins studied (streptolysin O and *C. perfringens* theta-toxin) bind to membrane cholesterol, whereas others (*C. perfringens* alpha-toxin and staphylococcal beta-toxin) hydrolyze membrane phospholipids or otherwise interact with phospholipids (e.g., streptolysin S) (2). None has been convincingly shown to react with membrane proteins. It is also significant that osmotic lysis is not suppressed by zinc ions. The facts suggest that zinc ions induce a reversible change in the state of the phospholipid bilayer. It seems probable that cell clumping by zinc ions also contributes to some extent to lysis inhibition because it can be shown that lytic agents liberate quantitatively less hemoglobin in the presence of a hemagglutinating agent than in its absence.

A clear precedent for the present findings is the observation that complement-mediated hemolysis is suppressed by zinc ions and by cupric ions as well (9, 12). Montgomery et al. (9) concluded that zinc does not affect "the membrane lysis step," but interferes with the binding and/or activation steps needed to form the complete complement complex. The interaction of Zn^{2+} and Cu^{2+} with complement itself was analyzed in detail by Yamamoto and Takahashi (12), who confirmed the findings of Montgomery et al. and who found, additionally, that these ions suppressed lysis also by acting directly on the erythrocyte membrane. The plasma membranes of L cells are stabilized by zinc (11), and the results of other studies show that zinc stabilizes lysosomes (6) and prevents release of his-

tamine from mast cells (8), probably through interaction with one or more components of the membrane. The precise mechanism of these stabilizing effects, although obscure, may be similar to that of the hemolysis inhibition reported here.

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